



Hudson, N., Celkova, L., Hopkins, A., Greene, C., Storti, F., Ozaki, E., Fahey, E., Theodoropoulou, S., Kenna, P. F., Humphries, M. M., Curtis, A. M., Demmons, E., Browne, A., Liddie, S., Lawrence, M. S., Grimm, C., Cahill, M. T., Humphries, P., Doyle, S. L., & Campbell, M. (2019). Dysregulated claudin-5 cycling in the inner retina causes retinal pigment epithelial cell atrophy. *JCI Insight*, 4(15), [130273]. <https://doi.org/10.1172/jci.insight.130273>

Peer reviewed version

Link to published version (if available):
[10.1172/jci.insight.130273](https://doi.org/10.1172/jci.insight.130273)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via American Society for Clinical Investigation at <https://insight.jci.org/articles/view/130273> . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

Title: Aberrant *BMAL1* dependent claudin-5 cycling in the inner retina causes retinal pigment epithelial cell atrophy

Natalie Hudson¹, Lucia Celkova¹, Chris Greene¹, Ema Ozaki², Erin Fahey², Sofia Theodoropoulou³, Paul F. Kenna¹, Marian M. Humphries¹, Annie Curtis⁴, Arvydas Maminishkis⁵, John J Callanan⁶, Pompei Bolfa⁶, Matthew S. Lawrence⁷, Pete Humphries¹, Sarah L. Doyle² and Matthew Campbell^{1*}

¹Smurfit Institute of Genetics, Trinity College Dublin, Dublin 2, Ireland.

²School of Medicine, Trinity College Dublin, Dublin 2, Ireland

³Academic Unit of Ophthalmology, School of Clinical Sciences, University of Bristol, Bristol, UK.

⁴Department of Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland (RCSI), 123 St. Stephens Green, Dublin 2, Ireland.

⁵Section on Epithelial and Retinal Physiology and Disease, National Eye Institute, Bethesda, MD, USA.

⁶Ross University School of Veterinary Medicine, Department of Biomedical Sciences Pathology, PO Box 334, Basseterre, Saint Kitts, West Indies.

⁷RxGen, Hamden, CT, USA

* To whom correspondence should be addressed

Dr. Matthew Campbell

Phone: 353 1 8961482

Fax: 353 1 8963848

Email: Matthew.Campbell@tcd.ie

Abstract

Age-related macular degeneration (AMD) is the leading cause of central retinal vision loss worldwide, with an estimated 1 in 10 people over the age of 55 showing early signs of the condition. While the end stage of wet AMD, namely choroidal neovascularization (CNV) can be controlled with regular intravitreal injection of anti-VEGF based medications, there are currently no forms of therapy available for the end stage of dry AMD, geographic atrophy (GA). Here, we show that the inner blood-retina barrier (iBRB) is highly dynamic and may play a central role in GA development. We have discovered that the gene *CLDN5*, that encodes the protein product, claudin-5, a tight junction component abundantly expressed at the iBRB, is tightly regulated by *BMAL1* and the circadian clock. Persistent suppression of claudin-5 expression in mice exposed to a cholesterol-enriched diet induced striking RPE cell atrophy and persistent targeted suppression of claudin-5 in the macular region of non-human primates induced RPE cell atrophy. These findings implicate an inner retina derived component to the early pathophysiological changes observed in AMD and we suggest that circadian regulation of claudin-5 at the iBRB allows for replenishment and renewal of components of photoreceptor outer segments that are phagocytosed by the retinal pigment epithelium (RPE) in a diurnal fashion every day. Our results also suggest that re-establishing dynamic claudin-5 cycling at the iBRB may represent a novel therapeutic target for the prevention and treatment of GA secondary to dry AMD.

Introduction

Age-related-macular-degeneration (AMD) is the leading cause of central retinal vision loss worldwide and it has been estimated that 10 % of people over the age of 55 already show early signs of disease. AMD presents in two forms that are generally referred to as either "dry" or "wet" AMD (1). The end stage of wet AMD is termed choroidal neovascularisation (CNV), where blood vessels sprout from the underlying choroidal vasculature disrupting the integrity of the retina and leading to acute vision loss. CNV occurs in approximately 1 in 10 AMD patients and can be treated with regular intraocular injection of antibodies or fusion proteins directed against vascular endothelial growth factor (VEGF) (2). The end stage of "dry" AMD is termed geographic atrophy (GA), where the retinal pigment epithelium (RPE) begins to degenerate in the region of the macula and can eventually lead to death of cone photoreceptor cells and eventually central retinal vision loss (3). There is currently no therapeutic for GA other than recommended lifestyle changes such as smoking cessation and dietary modification (4). Given the pervasive nature of AMD in the developed world and the wealth of research in this area, the underlying molecular pathology associated with GA development is still far from clear with the majority of studies to date focused on understanding aberrant signalling mechanisms in the RPE.

The major early pathological hallmark common to both dry and wet AMD is the accumulation of drusen between the RPE and the choroid, with reticular pseudodrusen depositing in the sub-retinal space in some patients with late AMD and GA. Drusen is composed of extracellular components including, but not limited to, amyloid- β , vitronectin, cholesterol and almost every complement component (5). These components have all previously been identified in several tissues including blood and photoreceptors and it is most likely that AMD is primarily a condition that involves aberrant clearance mechanisms. What is less clear however is the source of drusen and the dynamic events that occur during and after the diurnal shedding of photoreceptor outer segments (POS) and subsequent phagocytosis by the RPE (6).

On a daily basis, RPE cells phagocytose POS that are shed during renewal of photoreceptors (7). While some of the phagocytosed material is recycled to replenish essential components of the photoreceptors, other components in the material are exocytosed to the basolateral compartment of the RPE and are likely cleared by the systemic immune system via the choriocapillaris (8). This is a highly regulated process controlled by autophagic and phagocytic processes and dysfunctional rates of clearance are likely a significant contributing factor to drusen accumulation in some individuals as evidenced by residual-body build up in lysosomes observed in the RPE of AMD donor eyes (9, 10).

While drusen deposition and localisation differs from individual to individual, it is pertinent to consider that there is a considerable degree of symmetry in drusen patterning in each eye of a single individual (11, 12). This correlates with an equally high degree of interocular symmetry of retinal blood vessels between right and left eyes. These blood vessels which form the inner blood-retina barrier (iBRB) are critical to maintaining retinal homeostasis (13, 14). Endothelial cells that line these vessels have evolved tight junctions, a series of up to 30 interacting proteins that limit the paracellular space between endothelial cells to all but the smallest of molecules. As well as regulating the exchange of ions and macromolecules between the blood and the delicate neural microenvironment, these highly specialized endothelial cells protect the retina by restricting the entry of potentially damaging blood-borne agents such as neurotoxic chemicals, antibodies, pathogens, immune cells and anaphylatoxins. They also express a variety of transporters to control both the selective transport of nutrients into the retina and the efflux of metabolites and toxins from the retina via the transcellular pathway (15, 16, 17, 18).

Here, we provide evidence that one of the most enriched tight junction components at the iBRB, claudin-5 (19, 20), is under the control of the transcription factor *BMAL1* and the circadian clock. In addition, regulating its expression can very rapidly lead to a RPE atrophy phenotype in pre-clinical mouse and non-human primate models. Our data suggest that regulating the dynamic expression pattern of claudin-5 at the iBRB could confer control over the delivery and replenishment of the outer segments of photoreceptors and consequently control the burden of material consumed by the RPE on a daily basis. Our findings represent the first description of iBRB cycling related to one of the most common forms of central retinal blindness.

Results

The mouse and human retinal pigment epithelium (RPE) is phagocytically active during phases of outer segment shedding

On a daily basis, the effete tips of photoreceptors are shed and phagocytosed by the retinal pigment epithelium (RPE). This process happens in a diurnal manner and is stimulated by light (21), with active engulfment of outer segments visible in transmission electron microscope (TEM) images in the morning when compared to the evening (**Fig. 1a**). This process is accompanied by a surge in autophagic activity in the RPE of mice in the morning when compared to the evening (**Fig. 1b and c**) but can also be re-capitulated by stimulating primary human foetal RPE cells with purified outer segments (**Fig. 1d**). In tandem with an increase in transcript levels of autophagic machinery, the RPE cells *in vivo* appear to exhibit pro-inflammatory transcript changes during phases of active phagocytosis which peak in the morning and decrease in the evening (**Fig. 1e**). Given the daily phagocytosis by the RPE of material from the POS, it stands to reason that a replenishment process is required to allow for outer segment supplementation and restoration. This is clearly a dynamic process and while much outer segment material will be recycled within the RPE, a large amount of outer segment composition is dietary derived (lipids, cholesterol, docosahexaenoic acid) and must be obtained from the diet (**Fig 1b**). In this regard, the major focus to date has been on examining the integrity of the choriocapillaris endothelial cells that are proximal to the basolateral aspect of RPE cells (22, 23, 24). In donor AMD eyes with geographic atrophy (GA), recent evidence and indeed our own data, indicates a dropout of these CD31⁺ endothelial cells, in areas of atrophic RPE (**Fig. 1f**). In an effort to explore the integrity of the choriocapillaris endothelium in normal adult mouse eyes during phases of active/inactive phagocytosis, we observed no differences (**Fig. S1**) in the morphology/integrity of the choriocapillaris endothelial cells. However, we observed distinct differences in the density of the tight junctions associated with the endothelial cells of the inner retina, specifically those located in the outer plexiform layer (OPL) (**Fig. 1g**). While these vessels have not previously been reported to be dysfunctional in AMD, their proximity to the RPE, and their similarity to the microvasculature associated with the brain, suggests that they may be important for selective delivery of material to the neural retina (**Fig. 1h**).

Size-selective regulation of the inner blood retina barrier (iBRB) mediated by claudin-5 expression

Claudin-5 is the most enriched component of both the brain and retinal endothelial tight junctions (20). Indeed, its dose dependent expression is critical, with knockout mice dying within hours of birth (19, 25). Given our observation of distinct changes in the density of tight junctions associated with endothelial cells of the inner retina during the evening (non-phagocytic activity), we observed significant differences in the levels of expression of claudin-5 in the evening compared to the morning at both the protein and transcript level (**Fig. 2a**, **P = 0.0064, and **2b**, *P = 0.042). These transcriptional differences in claudin-5

expression were confirmed as circadian as opposed to diurnal as dark-adapted (DA) mice showed the same pattern of expression (**Fig. 2c**) as did mice that had their circadian rhythms inverted by altering their light cycle (**Fig. 2d**). While other tight junction components ZO-1 and occludin appeared to cycle dependent on the time of the day, these did not appear to be circadian in nature (**Fig. S2**).

Levels of claudin-5 were decreased in the microvasculature of the retina in the evening compared to the morning (**Fig. 2e and f**) and this was accompanied by the increased extravasation of a tracer molecule, (EZ-Link-Biotin, 660 Da MW), with enhanced tracer signal observed in mice in the OPL and inner/outer segment regions of the retina in the evening compared to the morning (**Fig. 2g**, *P = 0.0184). In mice examined using dynamic contrast-enhanced MRI (DCE-MRI), enhanced Gadolinium (800 Da MW) signal was observed in the retina in the evening when compared to the morning (**Fig. 2h**, **P < 0.001) and this was also manifested when fundus fluorescein angiograms (FFA) were conducted, with enhanced sodium fluorescein (376 Da MW) signal observed in the deeper vascular plexus in the evening when compared to the morning (**Fig. 2i**, **P < 0.001). This was also observed in other strains of mice including 129SV and CD1 mice (**Fig. S3**). Intriguingly, this circadian change in permeability appeared to be size selective, as no differences were observed in FFA analysis using a FITC-labelled dextran molecule of 4 kDa (FITC-Dextran) (**Fig. 2j**), suggesting claudin-5 cycling at the iBRB is regulating the passive diffusion of molecules below this molecular weight. Unusually, claudin-5 transcripts appeared to cycle in an identical manner in all tissues examined, with transcript levels being low in the evening and high in the morning in heart and liver (**Fig. 2k and l**).

Bmal-1 regulates claudin-5 levels in mouse and human retinal endothelial cells

Bmal-1 is a transcription factor that is an essential component of the circadian clock (26). The promoter regions of both mouse and human claudin-5 contain E-box sequences that act as Bmal-1 protein binding sites and can regulate claudin-5 expression (**Fig. 3a**). Indeed, in mice with a targeted disruption of *Bmal-1* expression in the vascular endothelium (*Bmal1^{ff}.Tie2-Cre*), levels of claudin-5 protein remained un-changed in the retinas of mice between the morning and evening (**Fig. 3b**) and this effect was observed at the transcript level even in mice lacking only a single *Bmal-1* allele in the retinal endothelium (**Fig. 3c**). The levels and pattern of expression of claudin-5 appeared the same in retinas of *Bmal1^{ff}.Tie2-Cre* mice (**Fig. 3d and e**) and there was no difference between FFA performed in these mice between the morning and evening (**Fig. 3f and g**). This phenotype was also observed in mice with a single *Bmal1* allele remaining, namely *Bmal1^{WT/ff}.Tie2-Cre* (**Fig. S4**). TEM images of the tight junctions associated with the endothelium of microvessels in the OPL showed no difference in the morning compared to the evening in *Bmal1^{ff}.Tie2-Cre* mice (**Fig. 3h**).

Cells *in vitro* do not have an entrained circadian rhythm; however, rhythmicity can be induced by serum shocking cells with 50% serum for 2 hours (27). Primary human retinal endothelial cells were exposed to a serum shock and claudin-5 levels were observed to cycle in manner identical to that observed in mice (**Fig. 3i**). However, RNAi-mediated suppression of the expression of *BMAL1* in human retinal endothelial cells prior to serum shock showed a cessation of claudin-5 cycling (**Fig. 3j and 3k**) that was accompanied by a decrease in Trans-Endothelial Electrical resistance (TEER) and a discontinuous pattern of expression at the plasma membrane of cells (**Fig. 3l and m**).

Targeted suppression of claudin-5 with a high cholesterol diet induces RPE atrophy

It is well established that environmental factors such as smoking and a high fat/cholesterol diet are major risk factors for AMD. In that regard, and to determine whether a ‘Western’

diet could induce RPE atrophy, we fed adult C57BL/6J mice a high fat diet for 3 months. These mice weighed almost double the weight of mice kept on a normal diet and were nearly 3 times heavier than mice kept on a calorie-restricted diet (**Fig. 4a and 4b**). While there was a decrease in the levels of claudin-5 observed in the retinas of mice on a high fat diet and a calorie restricted diet (**Fig. 4c**), there was no observable difference in the integrity of the RPE in these mice (**Fig. 4d**). Next, to re-capitulate a persistent phenotype similar to that observed in wild-type mice in the evening (*i.e.*, high sodium fluorescein signal), we sub-retinally injected an adeno-associated virus (AAV9) vector expressing claudin-5 shRNA under the control of a doxycycline-inducible promoter. A non-targeting (NT) AAV9 vector expressing doxycycline inducible shRNA targeting the non-mammalian gene luciferase was used as a control. Mice were fed a high cholesterol diet as described previously and doxycycline was supplemented in the drinking water. Within 3 weeks of constant claudin-5 suppression, enhanced sodium fluorescein extravasation was observed in mice fed a normal diet (**Fig. 4e**, top panels). However, in mice with suppressed claudin-5 fed a high cholesterol diet (28), enhanced fluorescein signal was observed in the deeper vascular plexus with apparent hyper-reflectivity of the underlying RPE (**Fig. 4e**, bottom panels). Analysis of histological sections of the retina showed enlarged, vesiculated/vacuolated RPE cells that contained a large amount of auto-fluorescent material (**Fig. 4f**). Analysis of flatmounts from eyes of these mice stained for the tight junction component ZO-1 showed regions of atrophic AMD where the underlying choroid was visible (**Fig. 4g**). As claudin-5 is not expressed in the RPE, and the AAV will transduce endothelial cells only within the retinal side of the outer blood-retina barrier (oBRB), this data is strongly suggestive of the passive paracellular diffusion of material from the systemic circulation towards the RPE. (**Fig. 4h**). In order to suppress claudin-5 in both the retina and the choriocapillaris, we developed a mouse model that would allow us to inducibly suppress claudin-5 throughout the whole body (**Fig 4i, j and k**). A similar phenotype was observed in these mice with a targeted “knockin” of claudin-5 shRNA, with enlarged and vesiculated RPE cells observed (**Fig 4l**) in mice with decreased levels of claudin-5 fed a cholesterol rich diet. The concomitant decrease in the RPE derived C-wave signal (**Fig. 4m**), suggests the phenotype is derived from the inner retinal vasculature as opposed to the choroidal vasculature.

Surprisingly, using an AAV based method to over-express claudin-5 in the retinal endothelium an identical phenotype was observed as when we suppressed expression, even in the absence of a cholesterol rich diet (**Fig 4n and o**), re-enforcing the concept that circadian entrained *BMAL1* dependent cycling of claudin-5 is critical to the health and integrity of the RPE.

Targeted suppression of claudin-5 in the primate retina can induce RPE atrophy

While the use of mouse models of disease has added greatly to our understanding of human conditions, it must be recognised that mice lack a cone rich macula similar to the human retina. Therefore, we designed an AAV expressing a doxycycline inducible shRNA targeting a homologous region of the human and African Green monkey claudin-5 mRNA. Three monkeys were injected sub-retinally in the macular region with this AAV and were supplemented with doxycycline in their diet. Within 2 weeks, we observed hyper-reflectivity in optical coherence tomography (OCT) measurements that were commensurate with atrophy observed in AMD patients and this was concomitant with enhanced sodium fluorescein leakage at the site of sub-retinal injection (**Fig 5a, b and c**). This phenotype was confirmed by OCT and histological analysis of retinal cryosections of this region (**Fig 5d and e**).

Discussion

Here, we describe for the first time, an inner retina derived contribution to RPE cell atrophy. Our series of observations could have major implications for the prevention of AMD in general and more specifically in the prevention of geographic atrophy (GA) secondary to dry AMD.

We have shown that the major iBRB associated tight junction component, claudin-5, cycles in a distinct circadian rhythm and is regulated by the circadian clock component *BMAL1*. Claudin-5 is one of the central mediators of paracellular passive diffusion of material from blood to retina and *vice-versa* and appears to be a critical factor in allowing for the daily passive diffusion of dietary material from the blood to the retina (13, 14). In this regard, we have shown that size-selective passive diffusion of systemically injected tracer molecules will move from the inner retinal vasculature into the retinal parenchyma and diffuse towards the outer retina and RPE. This is important as an inner retina derived supply of material to photoreceptor outer segments and RPE has not been described previously and may represent a critically important physiological process central to the development of AMD.

We have shown that chemical and genetic ablation of Bmal-1 function can prevent claudin-5 cycling and impact the integrity of primary human retinal endothelial cells. It is of note that *BMAL1* mediated circadian rhythmicity is dysregulated in the aged population (29, 30, 31) and it is tempting to suggest aberrant claudin-5 cycling in the human retina with age is a contributing factor to drusen accumulation and subsequent RPE atrophy.

It is well known that poor diet and smoking are major risk factors for AMD (32). However, we found that a high fat diet alone or indeed a calorie-restricted diet was not sufficient to induce an RPE phenotype in mice even after 15 weeks of dietary supplementation. Additionally, we found that simply producing more claudin-5 protein expression at the inner retina is insufficient to protect against RPE atrophy and actually induced RPE atrophy and retinal thinning. This suggests that the process of claudin-5 cycling at distinct times of the day, aligned with POS replenishment, is critical in maintaining RPE health and integrity.

For the treatment of CNV secondary to wet AMD and GA secondary to dry AMD, retinal translocation surgical procedures were previously undertaken in an effort to restore visual acuity in patients by surgically translocating the neural retina from the atrophic RPE affected area to a region of intact RPE cells. While these techniques represented advanced and technically demanding surgeries, they proved successful for a sub-group of patients with sub-foveal CNV. It was of note however, that up to 47% of patients exhibited RPE atrophy in the translocated region within 45 months of surgery, suggesting an inner retina derived process that could promote RPE cell stress and death (33, 34). Interestingly, iBRB breakdown has been related to a rare juvenile retinal condition, Coats' disease, where leakage of blood derived cholesterol crystals and lipid-laden macrophages infiltrate the sub-retinal space. The pathophysiology of this condition lends weight to an inner retinal vascular derived deposition of material towards the RPE (35). We therefore propose a circadian entrained cycling of the permeability of the inner retinal vasculature as a key contributing factor to a dynamic retinal interstitial kinesis that allows for replenishment of substrate to the photoreceptor's for outer segment renewal. In the ageing eye, with dysregulation of the circadian clock, we propose that this process ceases to be regulated and therefore will either deprive or overload the RPE with essential metabolites leading to drusen deposition and eventual RPE atrophy. Overall, our observations directly implicate claudin-5 as a key mediator of disease pathology in AMD and suggest that methods aimed at restoring the dynamic expression of this molecule in the ageing eye may prevent or indeed halt the progression of AMD.

Methods

Animal Experiments and Experimental groups

All studies carried out in the Smurfit Institute of Genetics in TCD adhere to the principles laid out by the internal ethics committee at TCD and all relevant national licences were obtained prior to commencement of all studies. C57/Bl6J were sourced from Jackson Laboratories and bred on-site at the Smurfit Institute of Genetics in TCD. Prior to experiments, all mice were kept on a 12 h light/dark cycle with lights on at 8 am and lights off at 8 pm. For experiments involving inversed cycles, mice were kept on a light/dark cycle with lights on at 8 pm and lights off at 8 am for a minimum of 3 weeks.

Transmission electron microscopy

Eyes were immersion fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 reagent overnight and washed 3 times with EM grade Phosphate buffered saline (PBS). Eyes were embedded in resin and 1µm sections were prepared using a diamond blade.

qPCR arrays

The mouse autophagy RT² Profiler PCR Array (Qiagen) was used to profile 84 autophagy-related genes. cDNA synthesis was carried out using the RT² First Strand Kit according to manufacturer's instructions. First, genomic DNA was removed with the genomic DNA elimination mix. For the reverse transcription step, the following reaction was prepared: 4 µl of 5X Buffer BC3, 1 µl Control P2, 2 µl RE3 Reverse Transcriptase Mix, 3 µl RNase-free water. 10 µl of the reverse transcription mix was mixed with 10 µl of the genomic DNA elimination mix and incubated for 15 min at 42°C and immediately for 5 min at 95°C. Each reaction was mixed with 91 µl of NF H₂O and placed on ice. RT-PCR was carried out using the RT² SYBR Green Mastermix by mixing the following: 1350 µl 2x RT² SYBR Green Mastermix, 102 µl cDNA synthesis reaction, 1248 µl RNase-free water. 25 µl was added to each well of the RT² Profiler PCR Array and the plate was sealed with optical adhesive film. The plate was centrifuged for 1 min at 1000 g and placed into a StepOne Plus™ Real-Time PCR instrument. RT-PCR conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. A melt-curve stage was added of: 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. The absolute quantitation method was used to quantify changes in mRNA levels between treatment groups.

Haemotoxylin and Eosin (H&E) staining

Paraffin sections were deparaffinised by dipping 10 times in xylene followed by rehydration in 10 dips each of 100 %, 90 % and 70 % ethanol. Slides were incubated in haemotoxylin solution for 6 min, rinsed in water, and then incubated in eosin solution for 2 min. Slides were rinsed in water and dehydrated by dipping 10 times in 70 %, 90 %, 100 % ethanol and once in xylene. Coverslips were placed on slides using Sub-X Mounting Medium. Slides were analysed under a light microscope (Olympus 1X81).

Indirect immunostaining of retinal flatmounts and retinal cryosections

Mouse retinal cryosections (12 µm thick) were fixed with methanol for 10 min at -20°C, washed twice in PBS and blocked/permeabilised with 5% normal goat serum (NGS), 0.5% Triton X-100 in 1X PBS for 30 min at room temperature. Sections were incubated with primary antibodies (rabbit anti-mouse claudin-5; rabbit anti-mouse ZO-1; rabbit anti-mouse CD31 (1:100)) at 4°C overnight. Sections were washed 3 x 5 min in PBS and incubated with fluorescently labelled secondary antibodies (goat anti-rabbit Cy3, goat anti-rabbit Cy2 (1:500)) for 2 h at room temperature followed by 3 x 5 min washes with PBS. To label blood vessels, sections were co-stained with Isolectin-IB4 Alexa Fluor 488 (1:300). All sections

were counterstained with Hoechst 33258 (1:10,000) for 30 seconds and mounted with Aqua Poly/Mount (Polysciences, Inc). Images were acquired using a Zeiss LSM 710 confocal laser scanning microscope. Identical acquisition settings were used to acquire images and image processing was performed in Adobe Photoshop and *ImageJ*.

Western blot analysis

Retinal tissue and cells were lysed in RIPA lysis buffer with phosphatase and protease inhibitors (Sigma), incubated on ice for 10 min and centrifuged at 12,000g at 4°C for 20 min. 10 µg of protein were resolved on 12 % SDS polyacrylamide gels. Proteins were transferred to PVDF membranes and blocked for 1 h at room temperature in 5 % non-fat milk and tris-buffered saline containing 0.1 % Tween-20 solution (TBST). Membranes were then incubated with primary antibodies against claudin-5 (1:1000), Bmal-1 (1:1000) and β-actin (1:5000) overnight at 4°C, washed 2 x 5 min in TBST and incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5000) for 2 h at room temperature. After 4 x 10 min washes in TBST, protein bands were visualised using enhanced chemiluminescence (ECL; Advansta) with an image analyser (LI-COR C-DiGit scanner). Densitometry was performed using ImageJ with protein bands of interest normalised to the loading control β-actin.

qPCR analysis

RT-PCR was carried out using a SensiFAST SYBR Hi-ROX Kit (Bioline) according to manufacturer's instructions. PCR was performed in a Step-One Plus™ Real-Time PCR instrument (Applied Biosystems). RT-PCR conditions were as follows: 95°C for 10 min; 37 cycles of 95°C for 15 s, 60°C for 30 s. A melt-curve stage was added: 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. The comparative C_T method was used to quantify changes in mRNA levels between treatment groups. The following primers were designed using *Primer3*.

Claudin-5	F 5' TTTCTTCTATGCGCAGTTGG 3'
	R 5' GCAGTTTGGTGCCTACTTCA 3'
Occludin	F 5' ACAGTCCAATGGCCTACTCC 3'
	R 5' ACTTCAGGCACCAGAGGTGT 3'
Zo-1	F 5' CCACCTCTGTCCAGCTCTTC 3'
	R 5' CACCGGAGTGATGGTTTCT 3'
Bmal-1	F 5' ATCAGCGACTTCATGTCTCC 3'
	R 5' CTCCCTTGCATTCTTGATCC 3'
β-actin	F 5' TCACCCACACTGTGCCCATCTACGA 3'
	R 5' CAGCGGAACCGCTCATTGCCAATGG 3'

Biotin permeability assay

Mice were injected iv through the tail vein with 2 mg/ml of EZ-Link™ Sulfo-NHS-Biotin (600 Da). Eyes were enucleated 5 min post-biotin injection and immersion fixed in 4 % paraformaldehyde (PFA, pH 7.4) overnight at 4°C. Eyes were cryoprotected by sequential incubations in 10%, 20%, 30% sucrose and frozen in OCT freezing media. Mouse retinal cryosections were incubated with Cy3-conjugated streptavidin (1:200) overnight at 4°C to label Sulfo-NHS-Biotin. Biotin leakage was visualised with a Zeiss LSM 710 confocal laser scanning microscope. Image analysis was performed in ImageJ by drawing regions of interest outside of blood vessels and measuring the mean pixel intensity.

Circadian/dark adaptation/inversion experiments

C57BL/6J, *Bmal1^{wt/wt}.Tie2-Cre*, *Bmal1^{wt/f}.Tie2-Cre* and *Bmal1^{f/f}.Tie2-Cre* mice housed on a normal light/dark cycle, inverted light/dark cycle or constant darkness for 24 h were sacrificed at 8AM and 8PM and retinas removed for protein and RNA analysis.

Circadian/dark adaptation/inversion experiments

C57BL/6J, *Bmal1^{wt/wt}.Tie2-Cre*, *Bmal1^{wt/f}.Tie2-Cre* and *Bmal1^{f/f}.Tie2-Cre* mice housed on a normal light/dark cycle, inverted light/dark cycle or constant darkness for 24 h were sacrificed at 8AM and 8PM and retinas were removed for protein and RNA analysis.

Magnetic Resonance Imaging (MRI)

iBRB integrity was assessed *in vivo* via MRI, using a dedicated small rodent 7 T MRI system located at TCD (www.neuroscience.tcd.ie/technologies/mri.php). Anaesthetised mice were physiologically monitored (ECG, respiration and temperature) and placed on an MRI-compatible support cradle, with a built-in system for maintaining the animal's body temperature at 37°C. The cradle was then positioned within the MRI scanner. Accurate positioning was ensured by acquiring an initial rapid pilot image, which was then used to ensure the correct geometry was scanned in all subsequent MRI experiments. T1 weighted MR images (resolution, 0.156 X 0.156 X 5 mm³; field of view: 20 X 20 X 17.9 mm³; matrix: 128 X 128 X 30; TR/TE: 500/2.7 ms; flip angle: 30°; number of averages: 3; acquisition time: 2 min, 24 sec; Repetitions: 12) following administration of 100 µl of a 1 in 3 dilution of Gd-DTPA (Gadolinium diethylene-triamine penta-acetic acid), administered via the tail vein. A quantitative assessment of iBRB integrity was also made using dynamic contrast enhanced (DCE) MRI, in which the passage of Gd-DTPA from the vasculature into the tissue was monitored over a period of 10 minutes post-injection (referred to above as "bolus chase analysis").

AAV2/9 design and generation

Short hairpin RNAs (shRNAs) designed to target transcripts derived from mouse claudin-5 were incorporated into AAV-2/9 vectors. Claudin-5 shRNA was cloned into the pSingle-tTS-shRNA (Clontech) vector, which uses a doxycycline inducible promoter. The plasmid incorporating the inducible system with claudin-5 shRNA was digested with BsrBi and BsrGI and ligated into the NotI site of the plasmid pAAV-MCS, such as to incorporate left and right AAV inverted terminal repeats (L-ITR and R-ITR). AAV-2/9 was then generated using a triple transfection system in a stably transfected Hek293 cell line for the generation of high-titre viruses (Vector BioLabs). In a similar manner, mouse claudin-5 cDNA was cloned into the genome of an AAV-2/9 vector for over-expression of claudin-5.

Cldn5 knockdown mouse generation

Mice were generated by standard blastocyst injection (Nagy et al., 2003) at Charles Rivers Laboratory in collaboration with Mirimus Inc. using their strict VAF/Elite™ health standards. A doxycycline-inducible claudin-5 shRNA was knocked in at the *Col1a1* locus on chromosome 11. Additionally, a CAG-lox-stop-lox-rtTA3-IRESmKate2 (CLR3K) allele was knocked in at the *Rosa26* locus on chromosome 6. Mice homozygous for the claudin-5 shRNA containing gene and the reverse tetracycline-controlled transactivator (rtTA) gene were crossed to Tie-2-Cre expressing animals for endothelial-specific expression of claudin-5 shRNA. Cre-negative littermate mice were used as controls. Doxycycline was administered to the drinking water (2 mg/ml in 2% sucrose solution) to activate shRNA expression and replenished three times a week.

FFA and OCT analysis

FFA and OCT was performed on mice using a Heidelberg Spectralis™ OCT (Heidelberg Engineering, Heidelberg, Germany). Pupils were dilated with 1% tropicamide and 2.5% phenylephrine and mice anaesthetized using a mixture of ketamine and domitor. Mice were intraperitoneally injected with sodium fluorescein (2 mg/ml) at same volume/weight (100µl/20g) to visualize the blood vessels. For size selectivity study, mice were injected with the same volume/weight of FITC-Dextran FD4 (Sigma, 2mg/m) in a similar fashion to sodium fluorescein. FFA images were captured from 2 min to 10 min every 30 s. FFA and OCT images were captured with a 30-degree angle of view. Heidelberg eye explorer version 1.7.1.0 was used to capture images. For quantification of microvessel permeability *ImageJ* was used for analysis.

RNAi experiment

Brain endothelial cells (bEnd.3) were transfected with 10 pmol non-targeting (NT) or Bmal-1 siRNA using Lipofectamine® 2000 transfection reagent (ThermoFisher Scientific) diluted in OptiMEM reduced serum media according to manufacturer's instructions. 48 h post-transfection, media was replaced with media containing 50 % foetal calf serum and cells were shocked for 2 h. Following serum shock, cells were incubated in serum-free media and protein lysates were collected every 4 h for 28 h.

Immunocytochemistry

Confluent bEnd.3 cells were washed twice in PBS and fixed with 4% PFA for 10 min at room temperature. Following fixation, cells were washed twice with PBS and blocked/permeabilised with 5% NGS, 0.05% Triton X-100 in PBS for 1 h at room temperature. Cells were incubated in primary antibody against claudin-5 (1:100) overnight at 4°C. Cells were washed three times with PBS and incubated with goat anti-rabbit Cy3 (1:500) secondary antibody for 2 h at room temperature, followed by four washes with PBS. Nuclei were stained with Hoechst 33258 (1:10,000). The slide was removed from the chamber and mounted with Aqua Poly/Mount (Polysciences, Inc) before visualisation by confocal laser scanning microscopy.

Transendothelial electrical resistance (TEER)

TEER measurements were performed on cells grown on 6.5 mm diameter 0.4 µm pore polyester membrane HTS transwell inserts (Corning Costar). TEER values were measured using an EVOM resistance meter fitted with “chopstick” electrodes. Prior to measurements, the apical and basolateral chambers were replaced with fresh medium. TEER values were recorded in triplicate and the average value of a no cell compartment was subtracted from the average of the triplicate measurements. TEER values were expressed as ohm*cm² (Ω.cm²).

High fat and high cholesterol diet study

C57Bl/6J mice were either given a high cholesterol diet (Envigo, TD.88051) or high fat (western) diet [Rodent diet with 45% kcal% fat, (Research Diets, Inc D12451)].

Electroretinography

Mice were dark-adapted overnight and prepared for electroretinography under dim red light. Pupils were dilated with 1 % cyclopentolate and 2.5 % phenylephrine. Animals were anaesthetised by i.p. injection of ketamine and xylazine. Standardised flashes of light were presented to the mice in a Ganzfeld bowl to ensure uniform retinal illumination. ERG responses were recorded simultaneously from both eyes using gold wire electrodes (Roland

Consulting GmbH) and Vidisic (Dr Mann Pharma, Germany) as a conducting agent and to maintain corneal hydration.

Non-human primate study

Pupils were be dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride drops. Subretinal injections of AAV expressing claudin-5shRNA under the control of a doxycycline inducible promoter were be performed under a surgical microscope in 3 African Green monkeys in a sterile surgical field under aseptic conditions. After placement of a lid speculum, a radial conjunctival peritomy incision was made in the superior temporal quadrant and a pars plana sclerotomy site marked on the sclera. A sclerotomy incision was be made at the marked location using a 25-gauge MVR knife. After an anterior chamber paracentesis by 30-gauge needle at the limbal cornea, and application of a contact lens coupling solution, a Machemer fundus contact lens was placed on the cornea. A 30-gauge anterior chamber cannula, employed as a sub-retinal injector, was then introduced through the superior temporal sclerotomy into the vitreous cavity under the operating microscope. The cannula was then be maneuvered so that it was apposed to the posterior temporal retinal surface at an oblique angle. The cannula was advanced to catch a fold of retina on the cannula tip and then advanced further to pierce the retina, avoiding injury to the underlying RPE and choroid. AAV was then injected in a small volume to confirm position and patency of the cannula tip. Once confirmed the total target volume of 100 µL of CLDN5 AAV-2/9 or control vector was injected, with monitoring of the injection under direct visualization through the microscope. The cannula was then removed from the retina and scleral insertion site. Tobramycin and dexamethasone ointment (Tobradex) was administered topically following the injection procedure.

Non-human primate (NHP) fluorescein angiography

Fundus colour photography and angiography was performed on anesthetized animals using a retinal camera (model TRC 50X; Topcon America Corp., Paramus, NJ) with Canon D4 digital imaging hardware and New Vision Fundus Image Analysis System software. Fluorescein angiography in the left eye was preceded by angiography in the right eye by 6 hours to allow adequate time for wash out of fluorescein between image series. Colour and red-free images were collected from each eye prior to infusion of 0.1 mL/kg 10% sodium fluorescein into the saphenous vein. A rapid series of angiogram images were collected from the posterior pole immediately after completion of the infusion for the first minute, and then at 2, 3 and 6 minutes. A single angiogram was also taken from the contralateral eye after collection of the 6-minute angiogram image to ensure inter-eye consistency in fluorescein levels in the optic nerve head and normal vasculature. Colour and red-free photographs and fluorescein angiograms were collected from each animal at 14 days post injection.

NHP OCT examinations

OCT was performed employing a Heidelberg Spectralis OCT Plus with eye tracking and HEYEX image capture and analysis software. Images were acquired from all the eyes at baseline, immediately post-injection and at 14 days (immediately pre-sacrifice). At all time points an overall volume scan of the entire macula was performed at a dense scan interval, encompassing the sub-retinal injection site where applicable.

NHP termination

On day 14 post-injection monkeys were euthanized by pentobarbital overdose while under ketamine sedation and globes enucleated. Globes were marked at the 12'00 position with

indelible ink prior to enucleation. Eyes were prepared embedding and sectioning by fixing in Davidson's fixative for 24 hours.

Statistical Analyses

Statistical analysis was performed using Student's T-test, with significance represented by a P value of ≤ 0.05 when 2 individual experimental groups were being analysed. For multiple comparisons, ANOVA was used with a Tukey-Kramer post-test and significance represented by a P value of ≤ 0.05 .

Acknowledgments

This work was supported by grants from Science Foundation Ireland (SFI), (12/YI/B2614 and 11/PI/1080), The Health Research Board of Ireland (HRB), the BrightFocus Foundation, and Enterprise Ireland (EI). The Campbell lab is also supported by an SFI Centres grant supported in part by a research grant from SFI under grant number 16/RC/3948 and co-funded under the European Regional Development fund by FutureNeuro industry partners. We thank Caroline Woods and Charles Murray for animal husbandry.

Conflict of Interest Statement

The authors declare no conflict of interest.

Author Contributions

NH: Conceived, designed and performed experiments and analysed data. LC: Performed FFA analysis and qPCR arrays. CG: Generated claudin-5 knockdown mice. EO: Performed OCT and FFA analysis. EF: performed western blot and qPCR analysis. ST: Performed EM analysis. PK: ERG analysis of mice. MH: Genotyped mice. AC: Assisted in design and implementation of circadian studies. AM: Isolated primary RPE cells. JJC: Conducted HFD study in mice. BP: Conducted HFD study in mice. ML: Design and implementation of non-human primate studies. PH: Design and management of study. SLD: Conceived experiments and wrote the paper. MC: Conceived experiments and wrote the paper.

References

1. Colijn JM, Buitendijk GHS, Prokofyeva E, Alves D, Cachulo ML, Khawaja AP, Cougnard-Gregoire A, Merle BMJ, Korb C, Erke MG, Bron A, Anastasopoulos E, Meester-Smoor MA, Segato T, Piermarocchi S, de Jong PTVM, Vingerling JR, Topouzis F, Creuzot-Garcher C, Bertelsen G, Pfeiffer N, Fletcher AE, Foster PJ, Silva R, Korobelnik JF, Delcourt C, Klaver CCW; EYE-RISK consortium; European Eye Epidemiology (E3) consortium. Prevalence of Age-Related Macular Degeneration in Europe: The Past and the Future. *Ophthalmology*. 2017 Dec;124(12):1753-1763.
2. Comparison of Age-related Macular Degeneration Treatments Trials (CATT) Research Group, Maguire MG, Martin DF, Ying GS, Jaffe GJ, Daniel E, Grunwald JE, Toth CA, Ferris FL 3rd, Fine SL. Five-Year Outcomes with Anti-Vascular Endothelial Growth Factor Treatment of Neovascular Age-Related Macular Degeneration: The Comparison of Age-Related Macular Degeneration Treatments Trials. *Ophthalmology*. 2016 Aug;123(8):1751-1761.
3. Fleckenstein M, Mitchell P, Freund KB, Sadda S, Holz FG, Brittain C, Henry EC, Ferrara D. The Progression of Geographic Atrophy Secondary to Age-Related Macular Degeneration. *Ophthalmology*. 2018 Mar;125(3):369-390.
4. Reynolds R, Rosner B, Seddon JM. Dietary omega-3 fatty acids, other fat intake, genetic susceptibility, and progression to incident geographic atrophy. *Ophthalmology*. 2013 May;120(5):1020-8.

5. Crabb JW, Miyagi M, Gu X, Shadrach K, West KA, Sakaguchi H, Kamei M, Hasan A, Yan L, Rayborn ME, Salomon RG, Hollyfield JG. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2002 Nov 12;99(23):14682-7.
6. De Jong PTVM. Elusive drusen and changing terminology of AMD. *Eye (Lond)*. 2018 Feb 9.
7. Yu B, Egbejimi A, Dharmat R, Xu P, Zhao Z, Long B, Miao H, Chen R, Wensel TG, Cai J, Chen Y. Phagocytosed photoreceptor outer segments activate mTORC1 in the retinal pigment epithelium. *Sci Signal*. 2018 May 29;11(532).
8. Boyle D, Tien LF, Cooper NG, Shepherd V & McLaughlin BJ (1991): A mannose receptor is involved in retinal phagocytosis. *Invest Ophthalmol Vis Sci* 32: 1464–1470.
9. Kim JY, Zhao H, Martinez J, Doggett TA, Kolesnikov AV, Tang PH, Ablonczy Z, Chan CC, Zhou Z, Green DR, Ferguson TA. Noncanonical autophagy promotes the visual cycle. *Cell*. 2013 Jul 18;154(2):365-76.
10. Yao J, Qiu Y, Frontera E, Jia L, Khan NW, Klionsky DJ, Ferguson TA, Thompson DA, Zacks DN. Inhibiting autophagy reduces retinal degeneration caused by protein misfolding. *Autophagy*. 2018 Jul 13:1-13.
11. Barondes M, Pauleikhoff D, Chisholm IC, Minassian D, Bird AC. Bilaterality of drusen. *Br J Ophthalmol*. 1990 Mar;74(3):180-2.
12. Mann SS, Rutishauser-Arnold Y, Peto T, Jenkins SA, Leung I, Xing W, Bird AC, Bunce C, Webster AR. The symmetry of phenotype between eyes of patients with early and late bilateral age-related macular degeneration (AMD). *Graefes Arch Clin Exp Ophthalmol*. 2011 Feb;249(2):209-14.
13. Campbell, M, Humphries, P. The blood-retina barrier. *Biology and Regulation of Blood-Tissue Barriers*, 70-84. M
14. Díaz-Coránguez M, Ramos C, Antonetti DA. The inner blood-retinal barrier: Cellular basis and development. *Vision Res*. 2017 Oct;139:123-137.
15. Wolburg, H. and A. Lippoldt, Tight junctions of the blood-brain barrier: development, composition and regulation. *Vascul Pharmacol*, 2002. 38(6): p. 323-37.
16. Bazzoni, G. and E. Dejana, Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev*, 2004. 84(3): p. 869-901.
17. Tsukita, S., M. Furuse, and M. Itoh, Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol*, 2001. 2(4): p. 285-93.
18. Vorbrodt, A.W. and D.H. Dobrogowska, Molecular anatomy of intercellular junctions in brain endothelial and epithelial barriers: electron microscopist's view. *Brain Res Brain Res Rev*, 2003. 42(3): p. 221-42.
19. Nitta, T., et al., Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol*, 2003. 161(3): p. 653-60.
20. Daneman R, Zhou L, Agalliu D, Cahoy JD, Kaushal A, Barres BA. The mouse blood-brain barrier transcriptome: a new resource for understanding the development and function of brain endothelial cells. *PLoS One*. 2010 Oct 29;5(10):e13741.
21. Laurent V, Sengupta A, Sánchez-Bretaña A, Hicks D, Tosini G. Melatonin signaling affects the timing in the daily rhythm of phagocytic activity by the retinal pigment epithelium. *Exp Eye Res*. 2017 Dec;165:90-95.
22. van Leeuwen EM, Emri E, Merle BMJ, Colijn JM, Kersten E, Cougnard-Gregoire A, Dammeier S, Meester-Smoor M, Pool FM, de Jong EK, Delcourt C, Rodriguez-Bocanegra E, Biarnés M, Luthert PJ, Ueffing M, Klaver CCW, Nogoceke E, den Hollander AI, Lengyel I. A new perspective on lipid research in age-related macular degeneration. *Prog Retin Eye Res*. 2018 May 4. pii: S1350-9462(17)30127-1.

23. Whitmore SS, Sohn EH, Chirco KR, Drack AV, Stone EM, Tucker BA, Mullins RF. Complement activation and choriocapillaris loss in early AMD: implications for pathophysiology and therapy. *Prog Retin Eye Res.* 2015 Mar;45:1-29.
24. Zeng S, Whitmore SS, Sohn EH, Riker MJ, Wiley LA, Scheetz TE, Stone EM, Tucker BA, Mullins RF. Molecular response of chorioretinal endothelial cells to complement injury: implications for macular degeneration. *J Pathol.* 2016 Feb;238(3):446-56.
25. Greene C, Kealy J, Humphries MM, Gong Y, Hou J, Hudson N, Cassidy LM, Martiniano R, Shashi V, Hooper SR, Grant GA, Kenna PF, Norris K, Callaghan CK, Islam MD, O'Mara SM, Najda Z, Campbell SG, Pachter JS, Thomas J, Williams NM, Humphries P, Murphy KC, Campbell M. Dose-dependent expression of claudin-5 is a modifying factor in schizophrenia. *Mol Psychiatry.* 2017 Oct 10.
26. Dierickx P, Van Laake LW, Geijsen N. Circadian clocks: from stem cells to tissue homeostasis and regeneration. *EMBO Rep.* 2018 Jan;19(1):18-28.
27. Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell.* 1998 Jun 12;93(6):929-37.
28. Toomey CB, Kelly U, Saban DR, Bowes Rickman C. Regulation of age-related macular degeneration-like pathology by complement factor H. *Proc Natl Acad Sci U S A.* 2015 Jun 9;112(23):E3040-9.
29. Wyse CA, Coogan AN. Impact of aging on diurnal expression patterns of CLOCK and BMAL1 in the mouse brain. *Brain Res.* 2010 Jun 14;1337:21-31.
30. Ando H, Ushijima K, Kumazaki M, Takamura T, Yokota N, Saito T, Irie S, Kaneko S, Fujimura A. Influence of age on clock gene expression in peripheral blood cells of healthy women. *J Gerontol A Biol Sci Med Sci.* 2010 Jan;65(1):9-13.
31. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes Dev.* 2006 Jul 15;20(14):1868-73.
32. Clemons, T.E.; Milton, R.C.; Klein, R.; Seddon, J.M.; Ferris, F.L., 3rd. Risk factors for the incidence of advanced age-related macular degeneration in the age-related eye disease study (areds) areds report no. 19. *Ophthalmology* 2005, 112, 533-539.
33. van Romunde SH, Polito A, Bertazzi L, Guerriero M, Pertile G. Long-Term Results of Full Macular Translocation for Choroidal Neovascularization in Age-Related Macular Degeneration. *Ophthalmology.* 2015 Jul;122(7):1366-74.
34. Takeuchi K, Kachi S, Iwata E, Ishikawa K, Terasaki H. Visual function 5 years or more after macular translocation surgery for myopic choroidal neovascularisation and age-related macular degeneration. *Eye (Lond).* 2012 Jan;26(1):51-60.
35. Ghorbanian S, Jaulim A, Chatziralli IP. Diagnosis and treatment of coats' disease: a review of the literature. *Ophthalmologica.* 2012;227(4):175-82.

Legends

Figure 1

a) Transmission electron microscopy (TEM) of mouse retina and retinal pigment epithelium (RPE) at 8AM (left panel) showing effete tips of rod photoreceptor cells being phagocytosed (red arrows) compared to 8PM (right panel). Choriocapillaris (CC), outer segments (OS). **b)** Schematic representation of increased autophagy and inflammatory gene transcripts during active phagocytosis in the morning (left panel) compared to the evening (right panel). Yellow arrows indicate directionality of material transfer across the luminal and abluminal surfaces of the RPE. **c)** Significantly increased levels at 8AM compared to 8PM of autophagy related genes caspase-8, Hsp90aa1, Gabarapl2, Bak1, Hsc70, Nfkb1, Gaa, Bad, Dram2, p27Kip1, Gabarap, Ctsd, Atg12, Rgs19 and Atg 5. (n = 3 independent experiments). **d)** Post stimulation of primary RPE cells with photoreceptor outer segments (POS) induced significant increases in P53, Nfkb1, Hsp90aa1, Sqstm1, Dapk1, Atg4c, Npc1, Atg16l2, Bcl2, Bid, Tgfb1, Ulk2, Casp8, Ctsb, Hspa8. **e)** Significant changes at 8AM compared to 8PM of the inflammation-related genes Panx1, Pea15a, Hsp90aa1, Tak1, Il1b, Tnf, Erk2, Sugt1, Ctsb, Jnk2, Nfkb1, Ikk1, Il18, Caspase-8 and Mal. (n = 3 independent experiments). **f)** CD31 (green) staining in non-diseased age matched donor eye (left panel) and advanced age related macular degeneration (AMD) with geographic atrophy (GA) (right panel). **g)** TEM images of retinal vessels in the mouse outer plexiform layer (OPL) at 8AM (left panels-top and bottom) and 8PM (right panels-top and bottom). Red arrow indicates endothelial cell tight junction. Lumen (L). **h)** Schematic of retinal blood vessel tight junction and retina histology.

Figure 2

a) Western blot analysis of claudin-5 expression at 8AM compared to 8PM. Densitometric analysis (right panel, $^{**}P = 0.0064$). (n = 5 for each time point). **b)** qPCR base analysis of claudin-5 transcript decreased at 8PM compared to 8AM ($^{*}P = 0.042$, n = 5 mice for both time points). **c)** Transcript levels decreased at 8PM compared to 8AM in mice dark-adapted (DA) for 24 hrs ($^{*}P = 0.0483$, n = 5 mice for DA-AM and n = 4 mice for DA-PM). **d)** Decreased levels of claudin-5 transcript in mice with inverted circadian rhythm ($^{*}P = 0.0235$, n = 3 mice for AM, n = 4 mice for inverted). **e)** Claudin-5 (red) levels in the retinas of mice at 8AM (left panel) compared to 8PM (right panel), outer nuclear layer (ONL), inner nuclear layer (INL), ganglion cell layer (GCL). **f)** High magnification images of claudin-5 (red) in the retinas of mice at 8AM (left panel) compared to 8PM (right panel). **g)** EZ-Link-Biotin (red) extravasation in the inner and outer segments of the photoreceptors at 8PM compared to 8AM. Retinal vasculature stained with isolectin IB4 (green). **h)** Significantly enhanced Gadolinium (800 Da MW) extravasation in the retina of mice at 8PM (right panel) compared to 8AM (left panel). Dynamic contrast-enhanced MRI (DCE-MRI)-right panel histogram, $^{***}P < 0.001$ (n = 5 mice for AM, n=4 mice for PM). **i)** Fundus fluorescein angiography (FFA) 3, 5 and 10 min post sodium fluorescein (MW: 376 Da) injection at 8AM (top panel) compared to 8PM (bottom panel), with enhanced signal at 8PM ($^{***}P < 0.001$, n = 5 mice for both time points). **j)** FFA analysis of FITC-Dextran-4 (FD-4) (MW: 4,000 Da) at 8AM (top panel) compared to 8PM (bottom panel) (n = 8 for both time points). **k)** Significantly decreased levels of claudin-5 transcript in the heart at 8PM ($^{*}P = 0.0393$) compared to 8AM and **i)** the liver ($^{*}P = 0.0386$) (n = 3 for both time points).

Figure 3

a) E-box sequences in the promoter region of mouse and human claudin-5. **b)** Claudin-5 expression at 8AM and 8PM in *Bmal1^{ff}.Tie2-Cre⁺* mice at 8AM compared to 8PM (n=5 for both time points, P= ns). **c)** Claudin-5 transcript levels in *WT.Cre⁺*, *Bmal1^{WT/ff}.Tie2.Cre⁺* and

Bmal1^{ff}Tie2.Cre⁺ mice. **d**) Claudin-5 expression in retinas of *Bmal1^{ff}Tie2.Cre⁺* at 8AM (left panel) compared to 8PM (right panel), **e**) higher magnification. **f**) Fundus fluorescein angiography (FFA) in *Bmal1^{ff}Tie2.Cre⁺* at 8AM (top panel) compared to 8PM showed **g**) no significant differences. (n = 12 mice for 8AM and n = 11 mice for 8PM) **h**) Transmission electron microscopy (TEM) analysis of tight junction integrity in *Bmal1^{ff}Tie2.Cre⁺* at 8AM compared to 8PM, Lumen (L). **i**) Claudin-5 expression in primary human retinal endothelial cells (HRECs) exposed to serum shock (50% serum) for 2 hours. **j**) Suppression efficacy of Bmal-1 siRNA on Bmal-1 transcript expression (*P = 0.0026, n = 3). **k**) Claudin-5 expression in bEnd3 cells with suppressed Bmal1 prior to serum shock exposure. **l**) Trans-endothelial electrical resistance (TEER) measurement in bEnd3 cells with Bmal1 suppressed (*P 0.0339, n = 5). **m**) Claudin-5 staining in bEnd3 cells post suppression of Bmal1. Claudin-5 (red), nuclei (green). Scale bar – 50 μ m.

Figure 4

a) Mice fed a calorie restricted (CR) diet, normal diet (ND) or high fat diet (HFD). **b**) Body weights of mice on CR, ND and HFD. **c**) Claudin-5 transcript levels in mouse retinas from mice on CR, ND or HFD (n = 5 mice for CR, n = 7 mice for ND/HFD, ANOVA *P=0.046). **d**) Retinal histology of mice fed a ND or a HFD. **e**) Fundus fluorescein angiography (FFA) in mice injected with a non-targeting shRNA AAV2/9 and fed a ND (top left panel), or HFD (bottom left panel). FFA analysis of mice injected sub-retinally with a claudin-5 targeting shRNA AAV2/9 and fed a ND (top right panel) or a HFD (bottom right panel). **f**) Retinal histology of mouse injected sub-retinally with a non-targeting shRNA AAV2/9 and fed a HFD (left panel) or claudin-5 targeting shRNA AAV2/9 and fed a HFD. DAPI (blue), autofluorescence (red), RPE: retinal pigment epithelium, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner plexiform layer. **g**) ZO-1 (green) staining in flatmounts of mice receiving AAV2/9.*NTshRNA* sub-retinally (left panel) or AAV2/9.*CL5shRNA* (right panel), (n = 4 mice). **h**) Schematic representation of retinal interstitial kinesis (RIK). Normal paracellular flux of blood-derived components towards the RPE (left panel). Aberrant flux of material from the inner retina inducing RPE atrophy. **i**) FFA analysis in *Cldn5.Tie2-Cre⁻* mice (left panel). FFA analysis in *Cldn5.Tie2-Cre⁺* mice (right panel). **j**) Quantitative analysis of fluorescein leakage in *Cldn5.Tie2-Cre⁺* mice (***P < 0.001, n = 7 mice for *Cldn5.Tie2-Cre⁻*, n = 8 mice for *Cldn5.Tie2-Cre⁺*). **k**) Claudin-5 (blue) expression in *Cldn5.Tie2-Cre⁻* mice (left panel) compared to *Cldn5.Tie2-Cre⁺* mice (right panel). IB4 (green). **l**) Retinal histology in *Cldn5.Tie2-Cre⁻* mice fed a HFD (left panel) compared to *Cldn5.Tie2-Cre⁺* mice fed a HFD (right panel), DAPI (blue). **m**) C-wave electroretinography analysis in *Cldn5.Tie2-Cre⁻* mice compared to *Cldn5.Tie2-Cre⁺* mice (n = 7 mice). **n**) FFA analysis in mice injected sub-retinally with saline (top panels) compared to mice injected sub-retinally with a CMV-mCldn5 expressing AAV2/9 (bottom panels). **o**) Optical coherence tomography (OCT) analysis of control mice (top panel) compared to mice injected with AAV2/9.*CMV.mCldn5* (n = 10 mice).

Figure 5

a) Retinal fundus photograph from African Green monkey pre-injection and **b**) 3 weeks post sub-retinal injection of claudin-5 targeting shRNA AAV2/9. **c**) Fundus fluorescein angiography (FFA) 3 weeks post sub-retinal injection of claudin-5 targeting shRNA AAV2/9. **d**) Optical coherence tomography (OCT) analysis of AAV2/9 injected eye. ILM: Inner limiting membrane, BM: Bruch's membrane). **e**) Histological analysis of retina injected with AAV2/9 expressing claudin-5 targeting shRNA (n = 3 monkeys).

Supplementary Figures

Supplementary Figure 1: **a)** Retinal pigment epithelial cell and choriocapillaris morphology at 8AM (left panel) and **b)** 8PM (right panel).

Supplementary Figure 2: **a)** Occludin transcript expression at 8AM compared to 8PM (* $P < 0.05$). **b)** Occludin expression following circadian inversion. **c)** ZO-1 expression at 8AM compared to 8PM (* $P < 0.05$) ($n = 3$ independent experiments). **d)** ZO-1 expression following circadian inversion.

Supplementary Figure 3: **a)** Fundus fluorescein angiography (FFA) analysis in 129 mice at 8AM (top panels) compared to 8PM (bottom panels). Significant sodium fluorescein signal observed at 8PM (right panel), *** $P < 0.001$, ($n = 10$ for 8AM, $n = 8$ for 8PM). **b)** FFA analysis in CD1 mice at 8AM (top panels) compared to 8PM (bottom panels). Significant sodium fluorescein signal observed at 8PM (right panel), *** $P < 0.001$, ($n = 5$).

Supplementary Figure 4: Fundus fluorescein angiography (FFA) analysis in *Bmal1*^{WT/f}.*Cre*⁺ mice at 8AM (top panels) compared to 8PM (bottom panels), with no significant differences observed (right panel). ($n = 20$ for both time points).